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10. Thank you!

Requirement of a Critical Period of Transcription for Induction of a Late Phase of LTP

Peter V. Nguyen,* Ted Abel, Eric R. Kandel

Repeated high-frequency trains of stimuli induce long-term potentiation (LTP) in the CA1 region that persists for up to 8 hours in hippocampal slices and for days in intact animals. This long time course has made LTP an attractive model for certain forms of long-term memory in the mammalian brain. A hallmark of long-term memory in the intact animal is a requirement for transcription, and thus whether the late phase of LTP (L-LTP) requires transcription was investigated here. With the use of different inhibitors, it was found in rat hippocampal slices that the induction of L-LTP [produced either by tetanic stimulation or by application of the cyclic adenosine monophosphate (cAMP) analog Sp-cAMPS (Sp–cyclic adenosine 3′,5′-monophosphorothioate)] was selectively prevented when transcription was blocked immediately after tetanization or during application of cAMP. As with behavioral memory, this requirement for transcription had a critical time window. Thus, the late phase of LTP in the CA1 region requires transcription during a critical period, perhaps because cAMP-inducible genes must be expressed during this period.

In both vertebrates and invertebrates, long-term memory differs from short-term memory in that it requires the expression of genes and the synthesis of proteins during a critical period (*1*–*3*). In invertebrates, this distinction is observed in certain forms of long-term synaptic facilitation that contribute to memory storage (*2*). The degree to which this molecular distinction between short- and long-term plasticity applies to the neuronal substrates for memory in the mammalian brain is less clear. To address this issue, we tested whether hippocampal LTP requires transcription.

Long-term potentiation is a persistent, activity-dependent form of synaptic modification that can be induced by brief, high-frequency stimulation of hippocampal neurons (*4*). Because LTP can last for days to weeks in the intact animal, it is an attractive model for certain types of long-term memory in the mammalian brain (*5*). Indeed, like behavioral memory, LTP in the CA1 region of hippocampal slices has distinct temporal phases (*6*). An early phase beginning immediately after tetanic stimulation and lasting 1 to 3 hours is induced by a single high-frequency train and does not require protein synthesis. A late phase (L-LTP) persists for at least 8 hours in hippocampal slices, requires three or more high-frequency trains for its induction, and is blocked by inhibitors of protein synthesis or cyclic adenosine monophosphate (cAMP)–dependent protein kinase A (PKA) (*7*, *8*).

A requirement for new protein synthesis often reflects an alteration in gene transcription, which suggests that L-LTP in hippocampal slices may also involve gene induction. Indeed, LTP in the dentate gyrus is accompanied by changes in the expression of specific immediate-early genes (*9*). However, the role, if any, of these inductive events is unclear because attempts to block the expression of LTP with inhibitors of transcription have been unsuccessful (*10*, *11*).

We examined the possibility that hippocampal LTP requires gene expression by using the following protocol. First, we carried out experiments in rat hippocampal slices, which avoids anesthesia and assures penetration of the inhibitor into pyramidal cells critical for LTP (*12*). Second, we initiated LTP with three stimulation trains, which induce a robust late and protein synthesis–dependent phase (*9*). Third, we used two transcriptional inhibitors, actinomycin D (ACT D) and 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB), which have distinct mechanisms of action (*13*). Fourth, we monitored the effectiveness of each inhibitor. Finally, we timed the application of the inhibitors so as to interfere optimally with the putative induction of immediate-response genes.

We first applied 25 μM ACT D for 2 hours immediately after three high-frequency

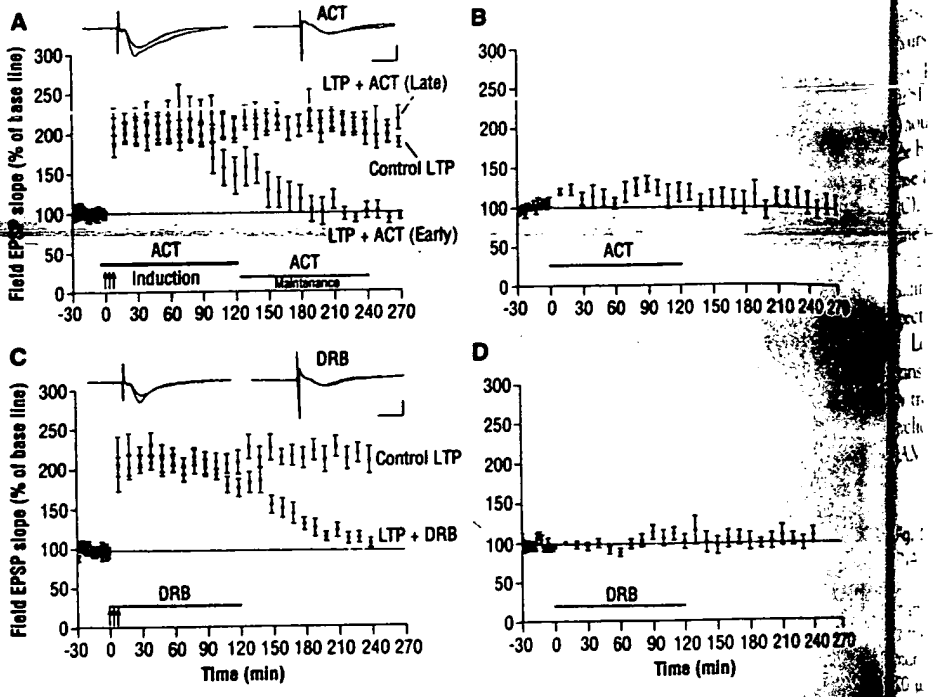

Fig. 1. Induction of L-LTP by tetanization is blocked by inhibitors of RNA synthesis. Shown are the time courses of changes in the slopes of field EPSPs measured from area CA1. (**A**) Application of ACT D (25 μM; horizontal bar) beginning at the first tetanus (arrows) blocked persistence of L-LTP ($n = 10$; $P < 0.05$ for all time points later than 100 min after the tetanus). Delayed application of ACT D, beginning 2 hours after the first tetanus (second horizontal bar; $n = 7$), had no effect on LTP maintenance. Sample EPSP traces were recorded from control ($n = 10$) and drug-treated slices 5 min before and 270 min after the first tetanus. Calibration bars: 1 mV, 5 ms. We induced LTP by giving three tetanic trains (arrows) (each train was 100 Hz for 1 s, with 5-min intervals between trains) at twice the test stimulus duration. This pattern of stimulation reliably induces a synaptic potentiation that persists for several hours (*8*). (**B**) Effect of ACT D on base line EPSP slopes ($n = 6$). (**C**) DRB (100 μM) applied for 2 hours beginning at the first tetanus prevented induction of L-LTP ($n = 11$; $P < 0.05$ for all time points later than 100 min after the tetanus). Sample field EPSP traces were recorded from control ($n = 8$) and DRB-treated slices 5 min before and 3 hours after the first tetanus. Calibration bars: 1 mV, 5 ms. (**D**) Effect of DRB (100 μM) on base line EPSP slopes ($n = 5$). Error bars indicate SEM.

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to the Schaffer collaterals and found as blocked induction of L-LTP. In con-ices, the mean field excitatory postsyn-potential (EPSP) slope 4 hours after the was 199 ± 16% (SEM) of the pre-base line values. By contrast, in drug-slices the mean EPSP slope was 102 ± the base line ($P < 0.05$, Mann-Whit-test). As with behavioral memory, this blockade had a specific time window: ACT D application was delayed 2 hours the first tetanus, there was no blockade LTP (Fig. 1A). The mean EPSP slope ed in treated slices 4 hours after the tanus (200 ± 14% of the base line was not significantly different from corded from control slices (199 ± 16%; .5). This concentration of ACT D ted [^{3}H]uridine incorporation into in slices by 71 ± 8% ($n = 6$) 2 hours drug addition; the inhibition reached 73 ($n = 8$) by 3 hours after addition (0 and after washout, respectively). RNA syn-inhibition subsided to 5 ± 8% ($n = 6$) hours after drug addition (5 hours after out) (Fig. 2).

We next used a different transcriptional itor, DRB, and obtained similar re-. Application of 100 μM DRB for 2 immediately after the first tetanus prevented induction of L-LTP. Field slopes measured in DRB-treated slices urs after the tetanus were 109 ± 6% of ase line compared to 212 ± 17% of the line in untreated slices ($P < 0.05$) (Fig. Like ACT D, DRB inhibited [^{3}H]uri-incorporation by 66 ± 8% ($n = 8$) and 3% ($n = 6$) 2 and 3 hours after drug ition (0 and 1 hour after washout), re-tively (Fig. 2).

Long-lasting potentiation of synaptic smission in area CA1 can also be elicited reatment of hippocampal slices with Sp-adenosine monophosphorothioate (Sp-MPS), a membrane-permeant cAMP ana-log that activates PKA (*8*, *14*). Because this cAMP-induced form of L-LTP is dependent on protein synthesis (8), we tested whether transcription might also be required for maintaining cAMP-induced potentiation. In control slices treated with Sp-cAMPS, field EPSPs were potentiated and reached a plateau of about 200% of pre-cAMP base line values by 90 min after washout of Sp-cAMPS. Elevated responses persisted for the remainder of the experiment (Fig. 3). When ACT D was introduced 30 min before Sp-cAMPS and left in the bath for 90 min, no facilitation was observed (Fig. 3). The mean field EPSP slope recorded 1 hour after washout of Sp-cAMPS in ACT D–treated slices was 83 ± 13% of pre-cAMP base line values. This value was not significantly different from the mean EPSP slope recorded before Sp-cAMPS addition ($P > 0.5$) and was significantly less than the increase produced by Sp-cAMPS in the absence of ACT D ($P < 0.05$).

The complete abolishment by ACT D of the potentiation produced by Sp-cAMPS (Fig. 3) suggests that the facilitation induced by Sp-cAMPS differs from that induced by tetanic stimulation of the Schaffer collaterals because Sp-cAMPS seems to induce a form of long-lasting potentiation that from its onset is entirely dependent on transcription. This would suggest the absence of an early, transcription-independent phase. It is interesting that anisomycin, a protein synthesis inhibitor, also completely blocks the onset of Sp-cAMPS–induced LTP (9). However, the initial transient synaptic depression induced by Sp-cAMPS (Fig. 3) (8) might mask an early facilitation so as to yield only an apparently slow-onset potentiation that is dependent on transcription.

Although ACT D and DRB blocked transcription and induction of L-LTP, these drugs may also produce nonspecific effects. For example, treatment with ACT D slightly increased base line EPSP slopes (Fig. 1B) (129 ± 10% of base line; $n = 6$), which reversed upon washout of the drug. This increase, however, cannot explain the observed decline of L-LTP in drug-treated, tetanized slices, because DRB did not produce this effect (Fig. 1D; $n = 5$). Thus, the use of two different transcription inhibitors that act through distinct mechanisms (*13*) allows us to conclude that the blockade of L-LTP induction was attributable to transcriptional inhibition.

The finding that the late phase of LTP requires transcription raises the question of whether synapse specificity is maintained during the late phase, despite the fact that the newly synthesized gene products can, in principle, be transported to inactive as well as active synapses. To address this, we examined synapse restrictions during L-LTP using two different pathways. We found that after repeated tetanization, the mean EPSP slope of the tetanized pathway was significantly higher throughout a 4-hour recording period ($P < 0.01$, $n = 9$) than were the mean slopes recorded from an unstimulated test pathway in the same slices (Fig. 4A). The test pathway showed no significant change in EPSP slope during the entire recording period ($P > 0.5$ for all time points in the test pathway of Fig. 4A). These results establish that the late phase of LTP is input-specific, like the early phase (*15*).

Using the same two-pathway protocol, we found that ACT D blocked expression of L-LTP in the tetanized pathway without significantly changing base line transmission in the unstimulated test pathway of the same slices (Fig. 4B). ACT D significantly

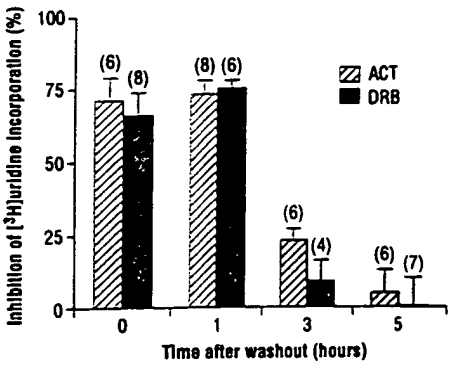

Fig. 2. ACT D (25 μM) and DRB (100 μM) inhibit synthesis in hippocampal slices. Rat hippocampal slices were incubated in an interface chamber, and after a 1-hour recovery period, drugs at the indicated concentration were added. One hour after drug addition, [^{3}H]uridine (NEN-Dupont) (5 μCi/ml) was introduced. After another hour in uridine, the drug and radioactive nucleotide were washed out by perfusion. Slices were removed at the indicated times, frozen on liquid N_2, and stored at −70°C. Extracts were prepared, and incorporation of [^{3}H]uridine into trichloroacetic acid (TCA)–precipitable material was assayed as described (*31*). We calculated the percentage inhibition at each time point by determining the ratio of TCA-precipitable radioactivity to the total radioactivity of control and experimental samples. In these experiments, the effects of both ACT D and DRB were reversed upon washout, an observation consistent with the low doses of drugs used and the long time course of continuous perfusion of the slices after drug treatment. Unlike ACT D, inhibition by DRB reversed more rapidly upon washout, with the level being only 17% ($n = 4$) 5 hours after DRB addition (3 hours after washout: $P < 0.05$, as compared to ACT D–treated slices). Numbers in parentheses indicate the number of hippocampal slices.

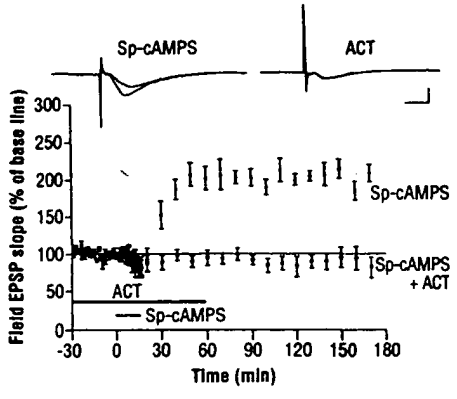

Fig. 3. Sp-cAMPS induces a long-lasting synaptic potentiation that is blocked by ACT D. A 15-min application of Sp-cAMPS (100 μM) produced a potentiation of synaptic transmission in control slices ($n = 6$) that was blocked by ACT D (25 μM, $n = 7$). ACT D was applied for 90 min, beginning 30 min before introduction of Sp-cAMPS. Both were applied by continuous perfusion. Sample field EPSP traces were recorded from control and ACT D–treated slices 5 min before and 2 hours after introduction of Sp-cAMPS. Calibration bars: 1 mV, 5 ms.

attenuated the mean EPSP slope measured 170 min after the tetanus and thereafter in the tetanized pathway ($P < 0.05$, $n = 7$, compared with the tetanized pathway in the control slices of Fig. 4A). These experiments provide additional evidence for a lack of effect of ACT D on base line transmission in area CA1.

Our results suggest a possible pathway for the flow of neuronal information for L-LTP from the membrane to the genome in the postsynaptic cell. Tetanization sufficient to induce L-LTP causes a transient increase in the concentration of cAMP in the pyramidal cells of area CA1, by means of a Ca^{2+}-stimulated activation of adenylyl cyclase (*8*, *16*, *17*). As the result of a tetanus, Ca^{2+} enters the pyramidal cell through *N*-methyl-D-aspartate (NMDA) receptor channels and perhaps also through voltage-gated Ca^{2+} channels (*18*). The rise in cAMP activates PKA, which alone or in combination with the Ca^{2+}-calmodulin–dependent protein kinase II (CaMKII) activated by Ca^{2+} influx might activate the cAMP-responsive element binding protein (CREB) (*19*) and perhaps other constitutive transcription factors. The finding of a critical time window is consistent with the recruitment, by CREB and other constitutive transcription factors, of immediate-response genes, many of which are expressed between 0.5 and 3 hours after LTP induction in the hippocampus (9). The products of immediate-early genes activated by LTP include effector proteins such as tissue plasminogen activator (tPA), growth factors, and a mitogen-activated protein kinase–specific phosphatase, as well as transcription factors such as *zif268* that may initiate a gene cascade (9, *20*). Because a similar molecular switch for long-term facilitation seems to be operative in invertebrates (*21*, *22*), our studies suggest that this mechanism for L-LTP may be quite general.

Finally, the transcriptional requirement for L-LTP suggests that although expression of the early phase is predominantly presynaptic (*23*), maintenance of the late phase of LTP may well involve the postsynaptic cell. LTP is induced postsynaptically by NMDA receptor–mediated Ca^{2+} influx (*24*), and a retrograde signal emanating from the postsynaptic cell is thought to trigger changes in the activated presynaptic terminals that lead to their long-lasting potentiation (*25*). Our finding that the late phase of LTP is input-specific (that is, restricted to the tetanized pathway) suggests that despite modifications of gene expression throughout the postsynaptic cell, the potentiation remains spatially confined to the postsynaptic dendrites directly apposed to the terminals of the activated fibers. Structural modifications at activated postsynaptic spines, mediated by dendrite-specific targeting of gene products (*26*, *27*), may underlie the input specificity of L-LTP.

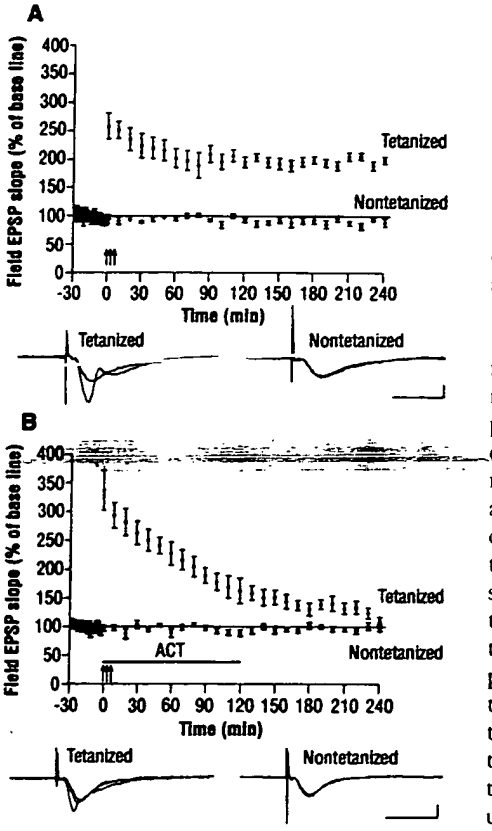

Fig. 4. The late phase of LTP is input-specific. (**A**) High-frequency stimulation of one pathway (tetanized) to CA1 produced a long-lasting increase in the EPSP slope, whereas an unstimulated test pathway (non-tetanized) in the same slices remained unpotentiated. Sample traces for both pathways were recorded from the same slice 10 min before and 3 hours after tetanization. (**B**) ACT D (25 μM) applied for 2 hours immediately after the first tetanus blocked expression of L-LTP without affecting base line transmission in an unstimulated test pathway. Sample traces were recorded from the same slice at 10 min before the tetanus (both pathways), 30 min after the tetanus (tetanized pathway only), and 3 hours after the tetanus (both pathways). Scale bars: 1 mV, 10 ms for both (A) and (B).

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11. This failure could reflect the lack of a transcriptional requirement for LTP. Alternatively, failure of the enzyme inhibitors to block LTP may also be due to one or more methodological factors. For example, most earlier experiments were carried out in intact animals that were anesthetized (*10*), a procedure that often blocks gene expression (*28*). Also, induction of the late, protein synthesis–dependent step of LTP requires repeated trains of tetanic stimulation (*29*). Because these stimulation parameters were not used in some of the earlier studies, they may have led to suboptimal L-LTP (*30*).
12. Transverse hippocampal slices (400 μm thick) from 5-week-old male Sprague-Dawley rats were prepared with conventional procedures and were maintained in an interface chamber at 28°C. Slices were subfused at a flow rate of 1 ml/min with an oxygenated (95% O_2/5% CO_2) saline solution consisting of 124.0 mM NaCl, 4.4 mM KCl, 1.3 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 10 mM glucose. Slices were allowed to recover for 90 min before recordings were attempted. A bipolar, nickel-chromium stimulating electrode was placed in the stratum radiatum layer of the CA1 region, and extracellular field potentials were recorded with a glass microelectrode (5 megohms, filled with perfusion saline) positioned in the stratum radiatum. Stimulation intensity was adjusted to give field EPSP amplitudes about 35% of maximum evoked amplitudes. Base line responses were evoked at 0.02 Hz. Five biphasic constant current pulses (0.2 Hz) were used for sampling at 2- and 10-min intervals after LTP induction. We measured the presynaptic fiber volley amplitude before and after tetanization and found no significant change in volley size after LTP induction. The mean sizes measured 10 min and 2, 3, and 4 hours after tetanization were 118 ± 15%, 99 ± 9%, 84 ± 16%, 115 ± 6%, and 122 ± 14% of pre-tetanus values, respectively ($P > 0.2$, $n = 8$). Hence, the potentiation induced under these conditions cannot be attributed to alterations in presynaptic fiber excitability. The EPSP slope (mV/ms) was measured from the average waveform acquired from five consecutive sweeps. Data were statistically evaluated with a two-tailed Mann-Whitney U test. Actinomycin D was prepared as a concentrated stock solution in 95% ethanol and then diluted to the appropriate final concentration in the perfusate (0.1% ethanol). Application of ethanol (0.05%) alone had no effect on LTP. DRB was dissolved in the perfusate by sonication. Both drugs were applied to slices by continuous subfusion for 2 hours.
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...rest of Motor Neuron Disease in *wobbler* Mice Cotreated with CNTF and BDNF

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...y neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) each ...ote the survival and differentiation of developing motor neurons, but do so through ...ct cellular signaling pathways. Administration of either factor alone has been shown ...w, but not to arrest, progression of motor neuron dysfunction in *wobbler* mice, an ...al model of motor neuron disease. Because CNTF and BDNF are known to synergize ...o and in ovo, the efficacy of CNTF and BDNF cotreatment was tested in the same ...al model. Subcutaneous injection of the two factors on alternate days was found to ... disease progression in *wobbler* mice for 1 month, as measured by several behav... physiological, and histological criteria.

...cytokine CNTF promotes the survival ...differentiation of developing motor ...ons in culture (*1*), rescues developing ...den motor neurons from death in ovo, ...etards axotomy-induced death of facial ...r motor neurons in the neonatal rat ... Administering CNTF to mice with in...ted neuromuscular deficits slows but ...not halt disease progression (*3*, *4*). ...F, a member of the neurotrophin fam... neurotrophic factors, promotes the ...val or differentiation of rat and chick... motor neurons in vitro and in vivo (*5*, ...nd synergistic interactions between ...F and CNTF have been observed in ...es with cultured rat neurons and in ovo ...).

...o assess the potential synergistic effects ...CNTF and BDNF in vivo, we adminis...ed these factors to *wobbler* mice, an ex...ively characterized animal model of ...or neuron disease relevant to amyotro... lateral sclerosis and spinal muscular ...phy (*8*, *9*). Mice with this inherited ...ait display forelimb muscle weakness be...ginning at 3 to 4 weeks of age, after which progressive paralysis, denervation atrophy, and contracture develop rapidly. Perikaryal vacuolar degeneration and neuron loss occur in the anterior horn of the spinal cord. Administration of CNTF alone (*4*) or (to a lesser extent) BDNF alone (*10*, *11*) has been shown to slow disease progression in these mice.

Upon diagnosis, 15 affected *wobbler* mice were randomly assigned to one of two treatment groups that received alternating doses of CNTF [1 mg per kilogram of body weight (mg/kg)] and BDNF (5 mg/kg) three times per week ($n = 8$) or vehicle solutions ($n = 7$) (*12*) for 4 weeks. All studies were blinded (*13*). Drug or vehicle was given by subcutaneous injection to the shaved lumbosacral area under halothane anesthesia. In a separate experiment, CNTF (1 mg/kg) or BDNF (5 mg/kg) alone was given five times per week to each of two groups of six *wobbler* mice for 4 weeks. All animals showed normal weight gain, and no adverse effects were seen in any of the treatment groups. The *wobbler* mice treated with vehicle solution showed a loss of grip strength (Fig. 1A), and paw position abnormalities progressed from mild to very severe (Fig. 1B). In contrast, the mice injected on alternate days with CNTF and BDNF showed no loss of mean grip strength, and paw position abnormalities showed no change. Several of

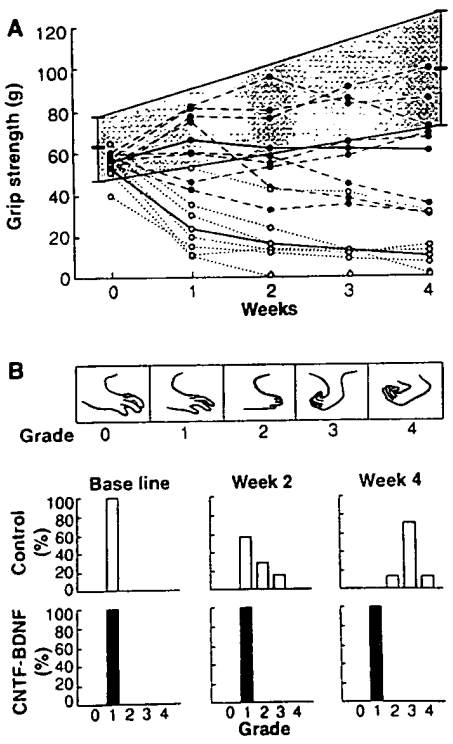

Fig. 1. Comparison of forelimb muscle function (**A**) and gradation of paw position abnormalities (**B**) in *wobbler* mice treated with vehicle solution ($n = 7$) or CNTF plus BDNF ($n = 8$). Shown in (A) are individual scores and means of grip strength measurements made at base line (3 to 4 weeks of age) and at weekly intervals for 4 weeks. Starting from the same base line, vehicle-treated mice (open circles) rapidly and progressively lost grip strength, whereas mice treated with CNTF and BDNF (solid circles) as a group maintained their initial grip strength. Grip strength differed significantly between the two groups at and after week 1 ($P < 0.0005$). The solid lines indicate the means for each group. Almost half the mice cotreated with CNTF and BDNF attained a grip strength that was comparable to that of 10 unaffected littermates, as shown in the shaded area (mean grip strength ± SD at 4 and 8 weeks of age). In (B), paw position abnormalities were graded as shown in the upper panel. All animals started as grade 1 (*12*). Over the course of 4 weeks, most of the vehicle-treated mice were judged to be grades 3 or 4, whereas mice cotreated with CNTF and BDNF did not go beyond grade 1.

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